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AGENT FOR ENHANCING HYALURONIC ACID PRODUCTIVITY
[Hiaruronsan sanseinou zoukyouzai]

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[Claims] /*

[Claim 1] An agent for enhancing hyaluronic acid productivity in a tissue or cell of a mammal, characterized in that the agent for enhancing hyaluronic acid productivity contains an extract of at least one plant belonging to the family Moraceae in an amount sufficient to enhance the production of the aforementioned hyaluronic acid.

[Claim 2] An agent for enhancing hyaluronic acid productivity cited in Claim 1, wherein a plant belonging to the family Moraceae is at least one species selected from a group comprising plants belonging to the genera Morus, Atrocarpus, Broussonetia, and Ficus.

[Claim 3] An agent for enhancing hyaluronic acid productivity cited in Claim 1, wherein a plant belonging to the genus Morus is the leaf or root bark of *Morus alba* L., the leaf, twig, fruit, or root bark of *Morus bombycis* Koidzumi, the leaf, fruit, stem bark or root bark of *Morus mongolica* Schneid., and the leaf, stem bark, or root bark of *Morus multicaulis*; wherein a plant belonging to the genus Atrocarpus is the flower or fruit of *Atrocarpus communis* Forst.; wherein a plant belonging to the genus Broussonetia is the leaf, fruit, or whole root of *Broussonetia kazinoki* Sieb. or the stem bark, leaf, or fruit of *Broussonetia papyrifera* L.; and wherein a plant belonging to the genus Ficus is the fruit, leaf, or stem of *Ficus carica* L., or the leaf, stem, or twig of *Ficus pumila*.

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* Claim and paragraph numbers correspond to those in the foreign text.

[Claim 4] An agent for enhancing hyaluronic acid productivity cited in any of Claims 1 to 3 wherein a plant extract is that which is extracted by water, a water-containing hydrophilic organic solvent, or water-immiscible organic solvent from the aforementioned plant material that has not been dried, or has been air-dried, heat-dried, or freeze-dried, for example.

[Claim 5] An agent for enhancing hyaluronic acid productivity cited in any of Claims 1 to 4, wherein the tissue or cell of a mammal is a human epidermal cell or a tissue containing said cell.

[Detailed Explanation of the Invention]

[0001] [Industrial Field of Application]

The present invention pertains to an agent for enhancing hyaluronic acid productivity in mammals, and particularly in human tissues or cells, containing as an active ingredient an extract of a plant belonging to the family Moraceae.

[0002] [Prior Art]

Substances that are known to have a hyaluronic acid synthesizing or productivity-enhancing effect, according to certain cell culturing experiments in mammals, include an extract of *Ulva pertusa*, a marine algae generally classified among the lower plants (JP 1994-009422A), and extracts from other marine algae, such as plants belonging to the families Ulvaceae, Gracilariaceae, and Gelidiaceae (JP 1995-101871A). It is suggested that these extracts cause an effect of promoting

hyaluronic acid synthesis, and bring about an activation of skin cells.

[0003] Perhaps, based upon the following findings and situation, it should be agreed that it is a fact that some sort of relationship exists between hyaluronic acid synthesis promotion ability and skin cell activation. Namely, hyaluronic acid is widely distributed *in vivo*, in skin, synovial fluid, vitreous body, and ligaments, for example; it plays an important role, for example, in skin with regards to cell adhesion, protection of cells, formation of skin tissue, tissue moisture maintenance, and maintenance of flexibility. Because hyaluronic acid declines in gonarthrosis and rheumatism, an injection solution is being used that has hyaluronic acid as an active ingredient, and in cataract surgical operations, hyaluronic acid is used as an aqueous chamber retention auxiliary. Separately, phenomena that occur as symptoms of aging skin include “lowering of wetness” and “lowering of tenseness,” which are accompanied by “wrinkles” and “sagging” for example. The causes of these phenomena have not yet been completely clarified, but it is thought that one factor is the decrease in production of hyaluronic acid by skin cells that accompanies aging, and the decrease in skin moisture content influences skin function, as reported in *Biochemica Biophysica Acta*, 279: 265 (1972); *Shougishi [English title: Japanese Journal of Society for Cosmetic Chemists] [sic] [actual English title: Journal of Society of Cosmetic Chemists of Japan]*, 15: 77 (1981); *Cell*

Structure and Function, 9: 357 (1984); *Carbohydrate Research*, 159: 127 (1987), for example.

[0004] When the aforementioned actual situation and findings are taken into consideration, even supposing that application to skin is a main object, it is desirable to offer a substance that is high in safety, that acts directly on skin cells, and that promotes production of hyaluronic acid by skin tissue more strongly. Accordingly, the object of the present invention is to provide a substance that is superior to conventional plant extracts in activity to enhancement of hyaluronic acid productivity, and that exhibit the aforementioned enhancement activity cells and tissue other than the skin.

[0005] [Means to Solve the Problems]

The inventors have, for example, focused on various culture cells derived from skin, and have conducted a series of investigations of the actions of various and divers compounds and substances, as well as compositions, on qualitative and quantitative changes over time of various glycosaminoglycans including hyaluronic acid, under [various] culture conditions.

[0006] As a result, the inventors discovered the fact that a plant extract of a plant belonging to the family Moraceae enhances hyaluronic acid productivity in mammalian tissue and cells, beginning with mulberry bark, which has conventionally come to be used as an active ingredient of a skin whitening cosmetic material (JP-B

(Tokuko) S52-44375), as an active ingredient for an external application to suppress melanin production used together with other ingredients (JP-A (Tokukai) H1-83009), as a 5-lipoxygenase-inhibition effect or hyaluronidase-inhibition effect material for an anti-inflammatory agent or a cardiovascular system medicine (JP-A (Tokukai) H3-68515), as an active ingredient of a cosmetic with continuous antibiotic or moisture-retention effect for the stratum corneum, used together with urea (JP-A (Tokukai) H6-24954), and as an active ingredient in skin whitening cosmetic materials together with other ingredients (e.g., JP-A (Tokukai) H6-107532, JP-A (Tokukai) H6-199646A, JP-A (Tokukai) H8-092055).

[0007] Unlike a melanin production suppression effect or tyrosinase activity inhibition effect [which are negative], the enhancement effect is characterized by the fact of positively promoting production of hyaluronic acid that shows various effects in a living organism. By making it possible to express such an effect, it then becomes possible, for example, to delay or improve dermal aging symptoms.

[0008] Therefore, an agent for enhancing hyaluronic acid productivity in mammalian tissue or cells is offered according to the present invention, there being contained an extract of at least one plant belonging to the family Moraceae, in particular from at least one species selected from a group comprising plants belonging to the genera Morus, Atrocarpus, Broussonetia, and Ficus, in an amount

sufficient to enhance the production of the aforementioned hyaluronic acid in the aforementioned tissues or cells.

[0009] The following plants and plant materials can be mentioned as specific examples of a plant belonging to a specific genus of the Moraceae that can be used in the present invention.

[0010] A plant belonging to the genus Morus includes: leaves and root bark of *Morus alba* L., leaves, twigs, fruit, and root bark of *Morus bombycis* Koidzumi, leaves, fruit, stem bark and root bark of *Morus mongolica* Schneid., and leaves, stem bark and root bark of *Morus multicaulis*, for example.

[0011] A plant belonging to the genus Atrocarpus includes: the flower and fruit of *Atrocarpus communis* Forst., for example. A plant belonging to the genus Broussonetia includes the leaf, fruit, and whole root of *Broussonetia kazinoki* Sieb., and the stem bark, leaf, and fruit of *Broussonetia papyrifera* L., for example. A plant belonging to the genus Ficus includes the fruit, leaf, and stem of *Ficus carica* L., and the leaf, stem, and twig of *Ficus pumila*, for example.

[0012] On an occasion of preparing an extract according to the present invention, it is acceptable for the aforementioned plant material to be in an undried form, but it is preferred, in terms of extraction efficiency, for the extract to be furnished after having been dried by a method such as air drying, heat drying, or freeze drying, for example. There are no particular restrictions on the

extraction method; it is possible to employ any extraction method customarily used in said technical field, and water, water-containing organic solvent, or organic solvent can be used as the extraction solvent.

[0013] The amount that is to be blended of plant extract for the agent for enhancing hyaluronic acid productivity according to the present invention is an amount that is sufficient to enable enhancement of hyaluronic acid productivity in mammalian tissue or cells, and particularly in human skin cells. A limit cannot be set on such an amount, because an optimal amount varies according to factors such as the age of the person using it and individual differences, but generally 0.0001 to 20% by weight, preferably 0.005 to 5% by weight is suitable when applied to skin, in terms of dry matter in the overall amount of formulation. An amount less than 0.0001% by weight is not sufficient to express the effect mentioned in the present invention, and an amount exceeding 20% by weight is undesirable [because of being unsuitable] for formulation. When an extract solution is blended, the amount to be used is 0.004 to 100% by volume, preferably 0.2 to 100% by volume. When the amount is under 0.004% by volume, the effects mentioned in the invention cannot be exhibited sufficiently.

[0014] In addition to the aforementioned extract that is contained as an active ingredient, the invented agent for enhancing hyaluronic acid productivity can contain, within a range that does

not adversely influence efficacy of the active ingredient, various carriers, diluents or auxiliaries, or other active compounds, respectively alone or in combination. In a case of formulation as a preparation for external application, for example, ingredients that are ordinarily used in external preparations such as surfactants, oils, alcohols, humectants, vitamins, thickeners, preservatives, antioxidants, chelating agents, pH-adjusting agents, fragrances, colorants, ultraviolet absorbers, ultraviolet-scattering agents, amino acids, skin function accelerators, hormones, skin activators, water, and the like can be used.

[0015] Specific examples include surfactants such as nonionic surfactants, anionic surfactants and cationic surfactants; hydrocarbons such as solid or liquid paraffin, crystal oils, ceresine, ozocerite and montan wax; vegetable or animal fats and oils and waxes such as silicone oils, olive oil, earth wax, carnauba wax and lanolin; fatty acids and esters thereof such as stearic acid, palmitic acid, oleic acid, glycerol monostearate, glycerol monooleate, isopropyl myristate and isopropyl stearate; esters between branched fatty acids and monohydric or polyhydric alcohols; alcohols such as ethyl alcohol, isopropyl alcohol, cetyl alcohol and palmityl alcohol; polyalcohols such as glycol, glycerin and sorbitol, for example, as well as esters thereof, and the like.

[0016] It is also possible to add [an additive] to the agent for enhancing hyaluronic acid productivity according to the present

invention, such as an amino acid, such as arginine, serine, or methionine; a vitamin, such as vitamin A acid, vitamin B6, ascorbic acid or a derivative thereof, a vitamin D derivative, vitamin E or a derivative thereof, or biotin; polysaccharides, cholesterols, pantothenic acid and derivatives thereof, glycyrrhizic acid and derivatives thereof, glycyrrhetinic acid and derivatives thereof, benzyl nicotinate and other nicotinic acid esters, ethyl paraben, butyl paraben and other preservatives, butyl hydroxytoluene, propyl gallate and other antioxidants, arbutin, kojic acid, and other skin beauty whiteners, placenta extract, cepharanthine and other skin function enhancers, estradiol and other estrogen preparations, retinol, alpha-hydroxylic acid and alkyl esters thereof, and the like.

[0017] The invented agent for enhancing hyaluronic acid productivity has a superior hyaluronic acid productivity enhancement effect, and has great safety when used with a living system, and thus it can be used for various uses such as the above-mentioned medicinal drugs, quasi-drugs, or cosmetics, for example. Any form thereof for use as an external composition for skin is acceptable, as long as it is a form capable of being applied to common integument, such as liquids, emulsions, creams, ointments, sticks, packs, pastes, and powders. The invented external preparation for skin can be applied by a method such as direct coating or by percutaneous administration by being stuck onto or sprayed onto skin.

[0018] The dosage of the invented agent for enhancing hyaluronic acid productivity cannot be clearly fixed, because it varies according to age, individual differences, symptoms, and other factors, but generally when used on a human being, it can be suitably used once daily, or 2 to 4 times daily, such that a dose ranging from 0.01 to 100 mg, preferably 0.1 to 50 mg, comes into contact or is adsorbed per 1 kg of body weight.

[0019] [Working Examples]

A method for producing the invented substance and its hyaluronic acid production enhancement effect is next explained in greater detail, with reference to working examples. The present invention is not limited by these examples.

[0020] (Production Method)

As a generic method for producing a plant extract used in the present invention, a plant material is subjected to extraction at a temperature, for example from 0 °C to 100 °C, using water, or a water-containing alcohol containing a lower alcohol such as methanol, ethanol, or isopropyl alcohol; a water-containing alcohol containing a polyalcohol such as propylene glycol or 1,3-butylene glycol; a hydrocarbon such as acetone, methyl ethyl ketone, acetonitrile, dimethyl sulfoxide, or hexane; or a chlorinated carbons such as chloroform, for example, giving [a plant extract]. The proportions of water and alcohol in a water-containing alcohol are preferably 1:99 to 100:0, more preferably 10:90 to 100:0.

[0021] Production Example 1

One liter of 50% aqueous ethanol solution was added to 100 g of dried *Morus alba* root bark, this was refluxed for extraction in a water bath of 80 °C for 4 hours, and then the extraction filtrate was concentrated and dried to give 17.8 g of *Morus alba* extract. In the same manner, filtrate or concentrates or dry powders were obtained from various plants, with the yields shown in Table I [sic], using extraction solvents in Table I.

[0022] Table I

Production Example	Plant Material	Extraction Solvent	Yield (g)
2	*Morus australis*	50% aqueous ethanol solution	28.3
3	*Morus mongolica Schneid.*	50% aqueous ethanol solution	29.2
4	*Morus multicaulis*	50% aqueous ethanol solution	21.4
5	*Artocarpus altilis*	50% aqueous ethanol solution	14.6
6	*Broussonetia kazinoki*	50% aqueous ethanol solution	13.8
7	*Broussonetia papyrifera*	50% aqueous ethanol solution	26.0
8	*Ficus carica*	30% aqueous ethanol solution	17.2
9	*Ficus pumila*	50% aqueous ethanol solution	23.0

(Test of hyaluronic acid production promotion)

Normal human epidermis-derived keratinocytes (NHEK) were cultured on serum free medium. These cells are often used to test physiological activities for keratinocytes of human skin, and are appropriate for testing hyaluronic acid productivity. 2.5 x 10^3 cells of NHEK were seeded per well (24 well plate) having a diameter of 15.6, serum free medium was used, and this was cultured for 3 days at

37 °C. After 3 days of culturing, the medium was replaced by a serum-free medium containing 0 to 2% of an extract shown in the list of Production Examples, and culturing was continued. After 7 days of culturing, the culture supernatants were taken and the hyaluronic acid concentrations were measured. The tests were conducted in triplicate, and average values were determined.

[0023] The hyaluronic acid concentration of each culture broth was measured according to a sandwich binding assay using a hyaluronic acid binding protein (HABP) (*Rinsho Byori [The Japanese Journal of Clinical Pathology]*, 36:536, 1991; *Biochemical Journal*, 309: 649-656, 1995; *Enshou [Japanese Journal of Inflammation]*, 16:97, 1996). The hyaluronic acid concentration in the culture broth of a comparative example that did not contain any test substance was measured at the same time. The hyaluronic acid concentration of an experimental example induced by the addition of a test substance was divided by the hyaluronic acid concentration of the comparative example to give hyaluronic acid productivity.

[0024] Experimental Example 1

Hyaluronic acid production enhancement ability was determined by first measuring the hyaluronic acid concentrations of the serum-free culture broths containing 0 to 0.1% of *Morus alba* extract of Production Example 1, and then dividing the average values by the average value of the hyaluronic acid concentration of a serum-free

culture broth not containing *Morus alba* extract (comparative example). The results are shown in the following Table 2.

[0025] Table 2

Test Substance	Concentration in broth (w/v)	Hyaluronic Acid Production Promotion Ability
Morus alba extract	0% (comparative example)	1.0
	0.005% (50 μg/ml)	1.2
	0.01% (100 μg/ml	1.6
	0.025% (250 μg/ml)	2.7
	0.05% (500 μg/ml)	4.2
	0.1% (1 mg/ml)	3.5

As shown above, the plant extracts containing the invented preparation showed a superior hyaluronic acid production promotion effect or hyaluronic acid production ability enhancement effect in human keratinocytes.

[0026] Next, classic formulation examples are given of cases wherein the invented hyaluronic acid synthesis ability is utilized in an external preparation for skin in order to bring about enhancement, particularly in skin cells or in a tissue containing said cells.

[0027] Formulation Example 1 (Ointment Preparation)

(1)	*Morus bombycis* extract (obtained in Production Ex. 2)	1.0%
(2)	Plastibase 50W	99.0
	Total	100.0%

Item (1) was kneaded together with Item (2), which comprises liquid paraffin (95%) and polyethylene (5%); this was deaerated under reduced pressure to give an ointment.

[0028] Formulation Example 2 (Cream Preparation)

A.	Cetanol	4.0%
	Vaseline	7.0
	Isopropyl myristate	8.0
	Squalane	12.0
	Dimethylpolysiloxane	3.0
	Glycerol monostearate	2.2
	POE(20) sorbitan monostearate	2.8
	Glycyrrhetinic acid stearate	0.02
	Ethylparaben	0.1
	Butylparaben	0.1
B.	Water phase	
	Morus alba extract (one obtained by Production Ex. 1)	0.1
	1,3-Butylene glycol	7.0
	Phenoxyethanol	0.2
	Ascorbic acid phosphate ester magnesium salt	4.0
	Purified water	Balance
	Total	100.0%

The *Morus alba* extract was dissolved under heating in 1,3-butylene glycol, then phenoxyethanol and ascorbic acid phosphate ester magnesium salt were added, and the mixture was held at 70 °C to give Phase B. Phase A [was prepared] as a solution by heating to 70 °C, and this was added to Phase B under stirring. A homomixer treatment was conducted to make the emulsified particles finer, and the mixture was rapidly cooled while stirring to give a cream.

[0029] [Effect of the Invention]

According to the present invention, a preparation is offered that causes enhancement of hyaluronic acid synthesis ability of keratinocytes derived from human skin and, furthermore, is able to recover or enhance the production ability of cells or tissues in which the ability to produce this has declined.